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PATENT APPLICATION
Docket No. 15892.9

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of		)
	William Richard Cross et al.	)
Serial No.:	10/522,371	) Art Unit) 1657
Filed:	January 25, 2005	)
Confirmation No.:	1386	)
For:	BIOMIMETIC UROTHELIUM	)
Examiner:	Laura J. Schuberg	)

DECLARATION OF DAVID JOHN TWEATS, PH.D. UNDER 37 C.F.R. & 1.132

Mail Stop AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, David John Tweats, Ph.D., hereby declare as follows:

1. I am personally knowledgeable of the facts stated herein.

2. I am a **NOT** a co-inventor of U.S. Patent Application Serial No. 10/522,371 ("Subject Application").

3. In the Spring of 2009, I undertook a one day paid consultancy with The University of York, to enable me to take part in a meeting organized by The University, although I am not and have never been an employee of The University of York, which has ownership via assignment in the Subject Application, and thereby I do not have any personal interest in the Subject Application.

4. I have extensive experience in the art of in vitro cultures of mammalian cells for use in toxicological screening and also in the limitations of current models. This allows me to evaluate the benefits of the ex vivo model of human urothelium and the stratification and differentiation thereof as applied in the Subject Application which is currently under evaluation. (See Appendix A: Curriculum Vitae of David John Tweats, PhD).'

5. I have reviewed and understand the Subject Application and the Cross and Zhang references.

6. I have reviewed the response to the Office Action being filed herewith, and I attest that the properties of rat urothelium related to stratification and differentiation are different enough from human urothelium such that data based on rat urothelium cannot be directly applied to human urothelium.

7. I attest that there has been a long-felt need for stratified, terminally-differentiated human urothelium for use in research and medical procedures. Currently, there is a lack of access to human urothelium ex vivo, and this lack of access is hampering studies on the response of this tissue to bladder specific toxins and carcinogens, and also of the physiological and molecular changes taking place in dysfunctional bladder epithelium. Accordingly, there is a pressing need for accurate models of the human urothelium, cultured in vitro. Normal, differentiated and stratified urothelium has a barrier function, dependent on molecular characteristics which are acquired during differentiation. As is common with many primary cell types, human urothelium cell differentiation is rapidly lost in normal culture of urothelium cells in serum free medium. A monolayer of amorphous undifferentiated human urothelium cells is formed with serum free medium.

8. I attest that the failure of others to identify a process, in view of cell culture techniques, for preparing ex vivo stratified, terminally-differentiated human urothelium is a significant reason that there has been a long-felt need for the same.

9. I attest that the current inventors and research team at The University of York have shown that a remarkable change takes place when human urothelium cells are propagated as recited in the claims of the Subject Application so as to produce stratified, terminally-differentiated human urothelium. The human urothelium cells are cultured in the presence of bovine serum and physiological levels of calcium ions in order to produce terminally-differentiated human urothelium. By processing the urothelium cells as described in the claims, the urothelium cells reacquire differentiated properties, and form a stratified, terminally-differentiated urothelium composed of the three layers: basal, intermediate, and superficial, as per the same tissue in vivo. This terminal-differentiation extends from the phenotypic level with the appearance of characteristic gene/protein markers, such as the uroplakins, to functional attributes of the tissue in vivo, including tight barrier properties (measured as high trans-epithelial resistance and low diffusive permeability to urea, water and dextran; Cross et al, 2005; Appendix B). Thus, a truely biomimetic human stratified and terminally-differentiated urothelial tissue can be formed in vitro as a result of this invention.

10. I attest that unlike the human stratified, terminally-differentiated urothelial cells that are prepared as claimed, rat cells proliferate in the presence of bovine serum, but do not differentiate (The University of York, data in Appendix C). Studies comparing rat and human urothelial cells in situ have shown that there are fundamental differences in cell cycle control between these species. Immunolocalization studies of peroxisome proliferatoractivated receptors (PPAR) and the retinoid X receptor (RXR) have shown that of the PPARs studied, human bladder cells express these receptors throughout the three layers, but in rats these receptors are expressed in only selected layers depending on the PPAR in question. Species differences in expression of both PPAR's and RXR were also seen between cultured rat and human urothelial cells (Chopra et al, 2008; Appendix D).

11. I attest that the invention made by the inventors is: isolating human urothelial cells; passing the isolated urothelial cells through a first nutrient medium containing serum and then redispersing the urothelial cells before they are added to a second medium containing serum in order to form stratified, terminally-differentiated human urothelium. The use of serum (e.g., bovine) to induce and maintain stratified, terminally-differentiated human urothelial cells in

culture so as to produce a truely biomimetic human urothelium is a significant advance in the art, such biomimetic human urothelium can be used in studies of bladder dysfunction and response to toxins.

12. I declare further that all statements made herein of my own knowledge are true and that all statements are made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this 28 day of September, 2009.

D J Tweats

David John Tweats, Ph.D

APPENDIX A

CURRICULUM VITAE OF DAVID JOHN TWEATS

EDUCATION

Crewe County Grammar School for Boys	1961-1968
Sheffield University	1968-1971
London University	1971-1974

QUALIFICATIONS AND FELLOWSHIPS (Academic and Professional)

10 Ordinary-Levels, 3 Advanced-Level UK national examinations.
Dual Honours Microbiology and Genetics. BSc First Class (1971). University of Sheffield.
Doctor of Philosophy (Medicine-Pharmacy), title of Thesis, "Genetic Interactions between R-factors and their Hosts"(1975). London University.
Fellow of the Institute of Biology (1989).
Fellow of the Royal College of Pathologists (1995).
Fellow of the UK Environmental Mutagen Society (UKEMS) (2008)

PRESENT ROLES

Preclinical Safety and Genetic Toxicology Consultant; Honorary Professor, Genetics Department, The Medical School, University of Wales, Swansea; Board member of Kirkstall Ltd (a company developing a bioreactor for in vitro toxicology/tissue engineering uses)

PREVIOUS POSITIONS

Vice President and UK Sites Head of Preclinical Safety Assessment UK, GlaxoSmithKline. Responsibility for 8 Departments and 200 staff. 2000-2002.

Director of UK Preclinical Safety Sciences, Glaxo Wellcome Research and Development, Park Road, Ware, Hertfordshire, SG12 0DP 1997-2000

International Head of Genetic and Reproductive Toxicology, GlaxoWellcome Research and Development, 1995-1997.

Department Head, Toxicology Department, Glaxo Research and Development 1995.

Department Head, Genetic and Reproductive Toxicology Department, Glaxo Research and Development Ltd, Ware, Herts, SG12 0DP. 1985-1994

Genetic Toxicology Section Head, Histopathology Department, Glaxo Group Research Ltd, Ware, Herts, SG12 0DP 1981-1985 (March)

Unit Head, Mutagenicity Unit, Histopathology Department, Glaxo Group Research Ltd, Harefield, Middlesex, August 1976-June 1981.

Research Demonstrator. Department of Genetics. University College of Swansea. October 1974 to July 1976.

SUMMARY OF TRACK RECORD

◊ Contributed to the generation and interpretation of regulatory toxicology data of most Glaxo marketed products 1977-2002 including cefuroxime; cefuroxime axetil; ceftazidime; ranitidine; fluticasone; salmeterol; sumatriptan, ondansetron, lamivudine etc.

◊ Introduced in vitro toxicology and supported an in vitro toxicology section within Glaxo as a tool in preclinical safety

◊ Influential in the harmonization of international preclinical safety guidelines for registration of pharmaceuticals through the ICH process.

◊ A member and former Chairman of the ICH Expert Working Group on Genetic Toxicology

◊ Former member and Co-chair of the European Federation of Pharmaceutical Industry Association (EFPIA) Ad Hoc Committee on Preclinical Safety.

◊ Former member of the UK Committee on Mutagens (1993-2002) that issued the national genetic toxicology guideline document in 2000.

◊ Successfully introduced genetic toxicology into Glaxo (mainly in vitro testing) and built up a team of scientists of international repute.

◊ Co-ordinator of a major EU Framework consortium on the integration of 'omic technologies into interpretation of preclinical safety studies (2004 – 2009).

◊ Member of the project team for the Drugs for Neglected Diseases initiative (DNDi) developing fexinidazole for treatment of African Sleeping Sickness (2004-ongoing)

◊ Seven years as a successful independent consultant working with large Pharma (e.g. Hoffmann-La Roche, sanofi-aventis; AstraZeneca) mid-sized Pharma companies (e.g. Almirall, Teva, Leo) and start up companies (e.g. ; Kirkstall Ltd, VireLogik, Gentronix); also worked with companies outside the Pharma industry including: Arysta, L'Oreal and Unilever.

◊ Continuous teaching experience in generic toxicology and preclinical safety, including courses at Swansea University, University of Surrey, Medicademy in Denmark and with TOPRA (Regulatory Affairs Organisation) in the UK and Czech Republic.

RELEVANT EXPERIENCE - EXTERNAL

◊ Honorary Professor, Department of Genetics, The School of Biological Sciences and The School of Medicine, University of Wales, Swansea (2000 - ongoing).

◊ Active referee for Mutagenesis, Mutation Research, Toxicology Letters and Regulatory Toxicology and Pharmacology (formerly 5 additional journals)

- ◊ Former member of the Editorial Boards for the journals 'Food and Chemical Toxicology' and 'Mutagenesis'.
- ◊ Industrial Supervisor for CASE award PhD students at Swansea and the University of London.
- ◊ External PhD examiner for the Universities of Bath, Swansea and Sussex.
- ◊ Invited lecturer for national and international scientific conferences, recently the Pharmaceutical Sciences World Congress, Amsterdam (March 2007); The Toxicology Forum, Brussels (October 2007), EUROTOX, Rhodes (October, 2008), International Conference on Environmental Mutagens, Florence, (August 2009), International Congress of Toxicology (IUTox), Barcelona (July, 2010). Regular chairperson at national and international conferences, including the ICEM in San Francisco (Sept 2005); BTS/UKEMS meeting at the University of Warwick (March, 2006), Gentromix user group (at the ICEM, 2009).

MEMBERSHIP OF RELEVANT COMMITTEES

Co-Chairman of the European Federation of Pharmaceutical Industry Associations (EFPIA) Ad Hoc Preclinical Safety Committee (2002 – 2004)
Member of the ABPI Toxicology Subcommittee 1992 – 2004
President of the United Kingdom Environmental Mutagen Society (UKEMS) 1994 - 1996.
Vice-President of UKEMS Committee 1992 - 1994
Secretary of the UKEMS Committee, 1984 - 1990.
Member of the UKEMS Education Subcommittee 2006 – ongoing
UK Councillor of the European Environmental Mutagen Society (EEMS) 1987 - 1991.
Member of the Presidential Nominating Committee for the EEMS 2002 – 2006
Chairman of ABPI Mutagenicity Working Party 1986 - 1995.
Chairman of the EFPIA Genotoxicity Working Party (1992 -1997).
A Chairman and EFPIA representative of the International Conference on Harmonization Expert Working Group on Genotoxicity 1991 - 1997.
Member of the Genetical Society Committee 1995 - 1998.
Member of the UK Department of Health Committee on Mutagens 1994 - 2003.

MEMBERSHIP OF SCIENTIFIC SOCIETIES

UKEMS/EEMS
US Environmental Mutagen Society
British Toxicology Society
Genetical Society
Royal College of Pathologists
Institute of Biology

FULL PUBLICATIONS

1. D.J.Tweats, R.J.Pinney and J.T.Smith. 1974. R-factor-mediated nuclease activity involved in thymineless elimination. J.Bacteriol. 118. 790-795.

2. D.J.Tweats. R.J Pinney. M.J.Thompson and J.T.Smith. 1976. R-factor-mediated resistance to ultraviolet light in strains of Escherichia coli deficient in known repair functions. J. Gen Microbiol., 93, 103-110.

3. J.M.Parry. D.J.Tweats and M. Al-Mossawi. 1976. Monitoring the marine environment for mutagens. Nature (London). 264. 538 -540.

4. D.J.Tweats and J.T.Smith. 1978. Interactions between the plasmid R6K and Escherichia coli with defective DNA polymerase I. Genet. Res. (Cambridge), 32. 25-35.

5. D.J.Tweats.M.H.L.Green. and W.J.Muriel. 1981.A differential killing assay for mutagens and carcinogens based on an improved repair deficient strain of Escherichia coli. Carcinogenesis. 2. 189-194.

6 D.G.Gatehouse and D.J.Tweats. 1982. Mutagen formation after the addition of nitrite to normal human gastric juice. Carcinogenesis 3. 597-598.

7. D.J.Tweats. 1984. The predictive value of batteries of short-term tests for carcinogens. Food Additives and Contaminants. 1. 189-197.

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CHAPTERS OF BOOKS

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18. Tweats, D.J. and Scales, M.D.C. (2002). Toxicity Testing. In 'The Textbook of Pharmaceutical Medicine' 4th Edition. Eds. J.P.Griffin and J. O'Grady, The Queens University of Belfast. Chapter 3. pp 128-169.

19. Tweats, DJ (2005). Preclinical Safety Tesring. In 'The Textbook of Pharmaceutical Medicine' 5th Edition, Eds J P. Griffin and J. O'Grady. The Queens University of Belfast. Chapter 3. pp 111-143.

REFEREES

1. Dr P Trennery, formerly Worldwide Director of Safety Assessment, GlaxoSmithKline, Park Rd., Ware, Herts, SG12 ODP.

2. Professor JM Parry, formerly Department of Genetics, The Medical School, University of Wales Swansea, Singleton Park: Swansea SA2 8PP

APPENDIX B

Am J Physiol Renal Physiol 289: F459–F468, 2005.
First published March 22, 2005; doi:10.1152/ajprenal.00040.2005.

A biomimetic tissue from cultured normal human urothelial cells: analysis of physiological function

W. R. Cross,[1,2] I. Eardley,[2] H. J. Leese,[3] and J. Southgate[1]
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Cross, W. R., I. Eardley, H. J. Leese, and J. Southgate. A biomimetic tissue from cultured normal human urothelial cells: analysis of physiological function. *Am J Physiol Renal Physiol* 289: F459–F468, 2005. First published March 22, 2005; doi:10.1152/ajprenal.00040.2005.—The urinary bladder and associated tract is lined by the urothelium. Once considered as just an impermeable epithelium, it is becoming evident that the urothelium not only functions as a volume-accommodating urinary barrier but has additional roles, including sensory signaling. Lack of access to normal human urothelium has hampered physiological investigation, and although cell culture systems have been developed, there has been a failure to demonstrate that normal human urothelial (NHU) cells grown in vitro retain the capacity to form a functional differentiated urothelium. The aim of this study was to develop a biomimetic human urothelium from NHU cell cultures. Urothelial cells isolated from normal human urothelium and serially propagated as monolayers in serum-free culture were homogeneous and adopted a proliferative, undifferentiated phenotype. In the presence of serum and physiological concentrations of calcium, these cells could be reproducibly induced to form stratified urothelia consisting of basal, intermediate, and superficial cells, with differential expression of cytokeratins and superficial tight junctions. Functionally, the neotissues showed characteristics of native urothelium, including high transepithelial electrical resistance (of >3,000 Ω·cm^2, apical membrane-restricted amiloride-sensitive sodium ion channels, basal expression of Na^+-K^+-ATPase, and low diffusive permeability to urea, water, and dextran. This model represents major progress in developing a biomimetic human urothelial culture model to explore molecular and functional relationships in normal and dysfunctional bladder physiology.

urothelium; cell culture; permeability; differentiation

THE URINARY BLADDER AND ASSOCIATED urinary tract are lined by the urothelium, an epithelium that is highly specialized to accommodate changes in bladder volume and provide a permeability barrier to urine (8). The urothelium has also been proposed to have a sensory role (9), dysregulation of which may be important in the pathogenesis of dysfunctional bladder syndromes, such as interstitial cystitis (41). Our current understanding of normal human urothelial cell physiology and specifically the relationship between morphological differentiation and functional specialization is hampered by the lack of suitable cell culture models (reviewed in Ref. 23).

The urothelium is a transitional epithelium and displays a regular architecture, increasing in morphological complexity from basal cells, through a variable number of intermediate cells, to the highly differentiated superficial or umbrella cells (20). The superficial cell layer is primarily responsible for providing the permeability barrier (30); the cells are interconnected by tight junctional complexes, which restrict paracellular ion transport and polarize the cell by limiting diffusion of transport proteins between the apical and basolateral membranes (12). In addition, superficial cells show a unique specialization of the apical plasma membrane, with thickened plaques of asymmetric unit membrane (AUM) decorating up to 90% of the luminal surface (16). These plaques are composed of a number of component proteins, the uroplakins (UPs) (7, 47, 48), which can be used as objective markers of terminal urothelial cytodifferentiation in many species, including man (31). The critical role of the AUM plaque in limiting transcellular permeability has been demonstrated in the UPIIIa$^{-/-}$ transgenic mouse, which developed a "leaky" urothelium in association with incomplete plaque formation (17, 18).

Consistent with its barrier properties, the transurothelial electrical resistance (TER) of the urothelium is one of the highest recorded for any tissue (2, 18, 21, 22, 24, 30). Although the urothelium is relatively impermeable, there is ion flux across the epithelium (8, 24). Sodium is the principal transported ion (25), by a mechanism that is modulated by a variety of molecular and physical factors (4, 10, 26, 46). It has been proposed that transurothelial sodium ion flux via mechanosensitive ion channels located in the apical membrane of the superficial cells may have a sensory role in normal micturition (9). Thus it is evident that there is an important relationship between molecular differentiation and function of the urothelium.

We have previously described a cell culture system to propagate normal human urothelial (NHU) cells (37, 38). In culture, NHU cells acquire a proliferative, regenerative phenotype but do not express markers of urothelial differentiation (20). NHU cells can be induced to express uroplakin genes (44, 45) and will form a stratified and partially differentiated urothelium when seeded on a decellularized urothelial stroma in organ culture (35). Although these observations suggest that cultured NHU cells retain the potential to undergo cytodifferentiation and are not compromised by propagation in vitro, it has yet to be demonstrated that the cells are capable of forming a functional barrier ex vivo. The purpose of this study was therefore to explore the capacity of in vitro propagated NHU cells to generate a functional barrier urothelium, with the longer term objective of using this model to explore the relationship between cytodifferentiation and pathophysiology.

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MATERIALS AND METHODS

Chemicals and Reagents

Unless specified otherwise, all chemicals were of analytical reagent grade or tissue culture grade, as appropriate, and were obtained from Sigma (Gillingham, UK).

Tissues

The collection of surgical specimens was approved by the relevant Local Research Ethics Committees and had full patient consent. Urothelial tissue samples were obtained at surgery from the upper and lower urinary tract of adult and pediatric patients with no history of urothelial dysplasia or neoplasia. Tissue samples were transported at room temperature from surgery in Hanks' balanced salt solution (HBSS, GIBCO, Paisley, UK) containing 10 mM HEPES, pH 7.6 (GIBCO), and 20 kallikrein-inhibiting units (KIU)/ml of aprotinin (Trasylol, Bayer Pharmaceuticals, Newbury, UK). To document tissue integrity on arrival at the laboratory and for comparison with subsequent cultured cells and tissues, representative portions of each sample were processed into paraffin wax for histology and immunohistochemistry. The remaining sample was cut into ~1-cm^2 pieces, placed into Ca^{2+}- and Mg^{2+}-free HBSS, containing 10 mM HEPES, pH 7.6, 20 KIU/ml Trasylol, and 0.3% (wt/vol) EDTA and incubated at 4°C overnight to release urothelial cell sheets. The isolated urothelium was used to establish finite NHU cell lines as previously described (37, 38).

Cell Culture

NHU cell cultures were established and maintained in keratinocyte serum-free medium (KSFM), containing recombinant epidermal growth factor and bovine pituitary extract at the manufacturer's recommended concentrations (Invitrogen, Paisley, UK) and supplemented with 30 ng/ml cholera toxin to improve cell plating and attachment (19). KSFM fully supplemented with the aforementioned factors will be referred to as KSFM complete (KSFMc). The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was replaced after 24 h and subsequently on alternate days for all experiments. Primary urothelial cells were propagated in Primaria tissue culture flasks (Becton Dickinson, Cowley, UK) and subcultured using a method described in detail elsewhere (37, 38).

The studies reported here are based on NHU cell lines established from 22 independent donors (1 bladder, 16 ureter, 5 renal pelvis, 15 men; mean age 48.2 ± 21.2 yr).

To develop a biomimetic urothelium (Cross WR and Southgate J. 2004; Biomimetic Urothelium; Patent Application WO2004/011630), urothelial cell cultures from NHU cell lines at *passages 1–3* were split into two groups, one set was maintained in KSFMc and the other was switched to KSFMc supplemented with 5% (vol/vol) fetal bovine serum (FBS; Harlan Seralab, Loughborough, UK). At confluence, the cells were harvested from Primaria flasks and seeded onto 1-cm^2 permeable Snapwell membranes (Costar, High Wycombe, UK) at a density of 1×10^6 cells/cm^2. After 24 h, the exogenous calcium concentration of the medium in one-half of both sets of cultures was increased from 0.09 mM (in KSFMc) to 2 mM, by use of a 1 M $CaCl_2$ stock solution (37); the addition of 5% FBS to KSFMc was found to raise the Ca^{2+} concentration to 0.2 mM, and this was taken into account when KSFMc containing both 5% FBS and 2.0 mM $CaCl_2$ was prepared.

NHU cell cultures were maintained in one of the following culture media: *1*) serum-free KSFMc (0.09 mM Ca^{2+}); *2*) serum-free KSFMc (2.0 mM Ca^{2+}); *3*) KSFMc supplemented with 5% FBS (0.2 mM Ca^{2+}); and *4*) KSFMc supplemented with 5% FBS (2.0 mM Ca^{2+}). Electrophysiological, permeability, and histological studies were performed on the cultures 7 days after they were seeded onto Snapwell membranes. Assessment of the mechanisms of transcellular ion transport was limited to cultures with a high transepithelial electrical resistance (TER; >1,000 Ω·cm^2).

NHU cell lines were routinely monitored for contamination by *Mycoplasma* spp. by scrutinizing for extranuclear fluorescence after staining cultures with the DNA-intercalating fluorochrome bisbenzimide (Hoechst 33258, Calbiochem, Nottingham, UK, see section below on immunofluorescence).

Functional Properties of NHU Cell Cultures

Electrophysiological properties of NHU cell cultures. The electrophysiological properties of urothelial cultures were monitored using a World Precision Instruments DCH01 electronic volt-ohmmeter (EVOM) and vertical modified Ussing chambers specifically designed to accept Snapwell membranes and gauze Ag-AgCl electrodes. All experiments were performed in modified Krebs solution (in mM: 118 NaCl, 25 $NaHCO_3$, 4.74 KCl, 1.19 $MgSO_4$, 1.17 $CaCl_2$, and 1 glucose) kept at a constant 37°C and equilibrated with 95% O_2-5% CO_2.

Measurement of transepithelial potential difference and short-circuit current. Urothelial cells cultured on Snapwell membranes were placed in vertical Ussing chambers with 5 ml modified Krebs solution in both apical and basal hemichambers. The spontaneous potential difference (V) and short-circuit current (I) across the urothelial cell layers (1-cm^2 culture membrane) were measured via electrodes connected to the EVOM and recorded on a computer, which was interfaced via an analog-to-digital converter. The TER was calculated from V and I using Ohm's law ($I = V/R$). The measured TER was corrected by subtracting the mean resistance of three blank Snapwell filters.

Assessment of transurothelial sodium ion transport. Transcellular sodium ion transport was investigated in urothelial cell cultures following determination of TER. Five microliters of the sodium ion channel inhibitor amiloride was added to the apical hemichamber (final concentration 5 μM-25 mM), and the transurothelial potential difference and short-circuit current were recorded until both parameters had stabilized. As the control, amiloride was added to the basal hemichamber.

The presence and membrane location of the ion pump Na^+-K^+-ATPase were delineated using ouabain. As above, the TER of the urothelial culture was determined initially, and both the transurothelial potential difference and short-circuit current were recorded throughout the experiment.

Determination of urea and water permeability of human urothelial cell cultures. Diffusive urea and water permeability coefficients were determined by measuring radioisotopic fluxes. After the TER of the urothelial culture had been assessed, 25 μl of [^{3}H]water (200 μCi/ml; Sigma) and 25 μl of [^{14}C]urea (200 μCi/ml; Amersham, Little Chalfont, UK) were added to the apical hemichamber. During the next 60 min, duplicate 100-μl aliquots were taken from both the apical and basal hemichambers at 15-min intervals and placed into 5-ml scintillation vials (PerkinElmer, Beaconsfield, UK) containing 4 ml Ultima Gold XR scintillation fluid (PerkinElmer). After sampling, the aliquoted volume was replaced with fresh Krebs solution and the TER was checked to confirm that the urothelial culture had not been physically disturbed. The number of counts of the individual isotopes within the samples was determined using a Packard Tri-carb 2700TR liquid scintillation counter.

The measured diffusive urea and water permeabilities (P_D) were calculated using the flux equation: $P_D = \Phi/(A\Delta C)$, where Φ is the flux of the isotope tracer across the membrane, calculated from the net increase of the tracer in the basal hemichamber, A is the area of the membrane, and ΔC is the concentration gradient of the respective across the membrane and is calculated from the mean concentration of the isotope in each chamber for the sampling period (30). In all flux measurements, corrections were made for sample dilution. To determine the permeability of the urothelial cultures, it was necessary to exclude the resistance to flow of water and urea exerted by the unstirred layers and the Snapwell membrane. The diffusive permea-

Table 1. *Primary and secondary antibodies*

Primary Antibodies	Host	Specificity	Source
Cytokeratins			
Ks 20.8	Mo	CK20	Cymbus Biotechnology
LP2K	Mo	CK19	Cancer Research UK
CY-90	Mo	CK18	Sigma
18	Mo	CK17	Sigma
LL001	Mo	CK14	Cancer Research UK
1C7	Mo	CK13	ICN
C51	Mo	CK8	Zymed
Tight junction components			
Anti-occludin	Rb	Occludin	Zymed
Anti-ZO-1	Rb	Zona occludin 1	Zymed
ZO1-1A12	Mo	Zona occludin 1	Zymed
Anti-claudin 1	Rb	Claudin 1	Zymed
Anti-claudin 2	Rb	Claudin 2	Zymed
2EJC1	Mo	Claudin 4	Zymed
Ion transporter			
6H (ex)	Mo	Na, K-ATPase	Novus Biologicals
Secondary Antibodies			
Fluorochrome			
Goat anti-rabbit Ig		Alexa Fluor 488	Molecular Probes
Goat anti-rabbit Ig		Alexa Fluor 594	Molecular Probes
Goat anti-mouse Ig		Alexa Fluor 488	Molecular Probes
Goat anti-mouse Ig		Alexa Fluor 594	Molecular Probes

Antibodies used for immunochemistry featuring monoclonal (Mo), and rabbit heteroantisera (Rb) and fluoroprobes are shown.

ability of the urothelial cultures ($P_{Durothelium}$) was calculated by measuring the mean permeability of three blank Snapwell membranes ($P_{Dinsert}$) using the following formula (30): $1/P_{Durothelium} = 1/P_{Dexperimental} - 1/P_{Dblanksnapwell}$.

Measurement of dextran permeability of human urothelial cell cultures. Permeability assays were performed using dextran (molecular weights 4,400 and 9,500) conjugated to fluorescein isothiocyanate (FITC). At the start of the experiments, the medium in the apical compartment of the Snapwell membrane was replaced with 500 μl of the appropriate growth medium containing one of the tracers at 1 mg/ml. The basal compartment was replaced with 1000 μl of tracer-free growth medium. The urothelial cells were returned to the incubator for 3 h, and then duplicate 250-μl samples were taken from the basal compartment and the amount of FITC-dextran was determined using a MFX microtiter plate fluorometer (Dynex, Worthing, UK). The amount of diffused dextran was calculated from a titration curve of known concentration (3.1–200 mg/ml).

Characterization of Native and Cultured Urothelial Cell Phenotypes

Paraffin wax and cryostat sections. Samples of the native tissue were fixed in 10% (vol/vol) formalin in PBS, dehydrated through graded alcohols, and embedded in paraffin wax. Five-micrometer sections were cut and stained with hematoxylin and eosin. Additionally, 5-mm^2 samples of tissue were embedded in Cryo-M-Bed compound (Bright, Burgdorf, Germany) before being frozen on card-ice, quenched in liquid nitrogen, and stored at −80°C. Five-micrometer cryosections were cut and collected onto 12-well Multitest slides (Hendley, Loughton, UK).

Immunofluorescent labeling of cryosections and cultured urothelial cells. Indirect immunofluorescence was performed as previously described (37). Cryostat sections and urothelial cell cultures grown on Snapwell membranes were labeled with polyclonal and monoclonal antibodies to cytokeratins (CKs), tight junction components, and a membrane transport-associated protein (Table 1). The Snapwell cultures were washed in PBS, fixed in a 1:1 mixture of methanol-acetone for 2 min, then air-dried. Cryosections were used unfixed. Urothelial cell cultures and tissue sections were incubated with appropriately diluted primary antibody for 1 h at room temperature. Excess unbound antibody was removed by two washes in PBS, followed by fixation in a 1:1 mixture of methanol-acetone for 2 min. After air-drying, the preturated fluorescence-conjugated secondary antibody (Table 1) was applied for 30 min. Slides were washed twice with 0.25% (vol/vol) Tween 20 (polyoxyethylene sorbitan monolaurate) in PBS, incubated for 5 min in a fluorescent DNA intercalating dye (0.1 μg/ml Hoechst 33258 or 2.5 μg/ml propidium iodide in PBS) to counterstain nuclei and rinsed in deionized water, before air-drying. Sections were mounted in glycerol containing 0.1% (wt/vol) p-phenylenediamine to prevent photobleaching. Tissue sections were viewed through an Olympus BX50 microscope equipped with wide-aperture oil-immersion objectives, epifluorescent illumination, dual and specific FITC, and Texas red filters (Olympus Southall, UK). Labeled urothelial cell cultures were also analyzed using a Nikon Bio-Rad laser confocal microscope equipped with an argon laser. Omission of primary antibodies from the labeling protocol served as negative controls.

Immunoblotting. Cell cultures were lysed directly into reducing SDS electrophoresis sample buffer, resolved on 8–16% SDS polyacrylamide gradient gels, and electrotransferred onto nitrocellulose membranes. Membranes were incubated with titrated primary monoclonal antibodies against CK13 or CK14 (Table 1) for 16 h at 4°C. Bound antibody was detected with goat anti-mouse IgG Alexa 680 (Molecular Probes, Paisley, UK) and visualized on a LI-COR Odyssey infrared scanner (LI-COR Biosciences UK, Cambridge, UK). To check loading, blots were stripped and reprobed with anti-β-actin monoclonal antibody (Sigma) followed by secondary anti-mouse IgG Alexa 680 (Molecular Probes) and detected as above.

Transmission electron microscopy. Samples of freshly isolated tissue and urothelial cell cultures propagated on Snapwell filters were fixed in 0.1 M phosphate buffer (pH 7.2) containing 4% (wt/vol) paraformaldehyde and 2.5% (wt/vol) glutaraldehyde for 1–2 h at room temperature. The samples were washed in phosphate buffer and postfixed for 1 h at 2°C with 1% (wt/vol) osmium tetroxide in 0.1 M phosphate buffer. Specimens were dehydrated through graded ethanols, cleared in propylene oxide, and impregnated with increasing ratios of Araldite-Polabed resin/propylene oxide with final embedding in 100% Araldite-Polabed resin. Seventy-nanometer sections were prepared on gold mesh grids, stained with 2% uranyl acetate, followed by 0.25% lead citrate in 0.4% NaOH. Specimens were viewed at 80 kV in a Jeol 1200EX electron microscope (Joel, Garden City, UK).

Scanning electron microscopy. Urothelial cell cultures on Snapwell filters were fixed as for transmission electron microscopy, dehydrated through graded ethanols, and critical point dried. Coated specimens were examined in a Hitachi S-2400 scanning electron microscope at various magnifications, with an accelerating voltage of 5 kV. Micrographs were taken using a Pentax A3 Date 5 camera on Kodak T-max film.

Statistics

Means and standard deviations or standard errors were used as descriptive statistics. For determination of statistical significance, Instat 3 software (GraphPad, San Diego, CA) was used for analysis of variance using the Kruskal-Wallis test (nonparametric ANOVA). A P value <0.05 was regarded as statistically significant.

RESULTS

Phenotypic Properties of Cultured Human Urothelial Cells

Urothelial cell cultures derived from the renal pelvis, ureter and bladder of different donors displayed similar growth characteristics, morphological, and immunocytochemical charac-

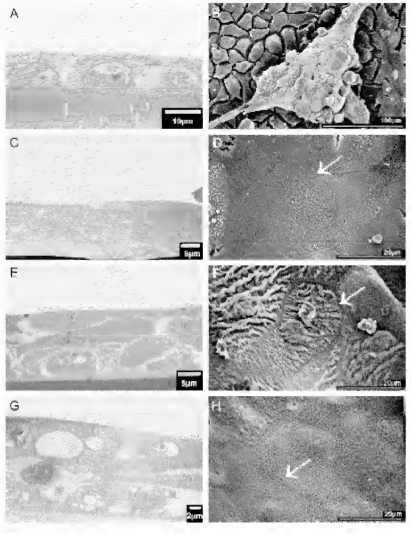


Fig. 1. Transmission and scanning electron micrographs of cultured urothelial cells. Electron micrographs of urothelial cells cultured in Keratinocyte serum-free medium complete (KSFMc; *A* and *B*). KSFMc supplemented with calcium (*C* and *D*), FBS (*E* and *F*), and CDS plus calcium (*G* and *H*) are shown. In KSFMc, the urothelial cells predominantly formed a monolayer with occasional islands of stratification. At sites of stratification, in all media, the superficial cells were interconnected by tight junctions (white arrows). The pronounced intercellular gaps seen in the cultures generated in the unsupplemented medium suggest that these cells had poor quality cell-cell adhesions relative to the cells propagated in the supplemented media.

teristics irrespective of the age, sex, and clinical status of the individual.

Primary and passaged urothelial cell cultures established and propagated in KSFMc formed monolayers when grown on Primaria tissue culture plastic. When seeded onto Snapwell membranes, the urothelial cells maintained in KSFMc continued to grow as monolayers, but after 24–48 h, small islands of cellular stratification also developed (Fig. 1*B*). Urothelial cell cultures transferred onto Snapwell membranes and switched to medium supplemented with calcium and/or FBS exhibited cellular stratification of between 3 and 7 cell layers (Fig. 1, *C*, *E*, and *G*).

At sites of urothelial stratification, prominent intercellular tight junctions were visible between the superficial cells, irrespective of whether the cells had been propagated in KSFMc or KSFMc supplemented with calcium and/or FBS (Fig. 1, *D*, *F*, and *H*). Reflective of the native urothelium, immunocytochemical studies demonstrated that the tight junctions consisted of the cortical protein zonal occludens-1 and the integral proteins occludin and claudin 4 (Fig. 2). Claudin 1 was expressed at the cell border at sites of urothelial stratification (Fig. 2). In addition, the transport protein Na^+-K^+-ATPase was located inferior to the tight junctions and restricted to the basolateral membrane of the superficial cells (data not shown).

Ultrastructurally, the apical membrane of cultured urothelial cells was flat with numerous small microvilli, irrespective of the medium in which the cells had been propagated. There were no obvious concave thickened regions, characteristic of the AUM plaques of superficial urothelium in situ (Fig. 1*A*, *C*, *E*, and *G*).

Urothelial cytodifferentiation. The CK expression profile was used to determine the stage of maturation of the urothelial

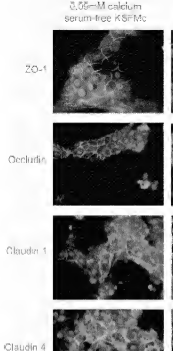


Fig. 2. Expression of tight junction proteins in cultured urothelium. Laser confocal micrographs of cultured urothelial cells labeled for tight junction components (rows) and including ZO-1, occludin, claudin 1, and claudin 4 (green) and propidium iodide-stained cell nuclei (red) are shown. Urothelial cells propagated in KSFMc (KSFMc supplemented with calcium (data not shown), FBS (data not shown), and FBS plus calcium exhibited tight junctions between the superficial cells at apices of stratification. Scale bar = 50 μm.

cultures (36). As in native urothelium, NHU cells grown on Snapwell membranes were positive for CK8, CK13, CK17, CK18, and CK19, irrespective of the culture medium in which they were propagated (Table 2). None of the cultured urothelial cells expressed CK20. However, the composition of the culture medium affected the proportion of cells expressing CK13 and CK14 (Fig. 3, *A* and *B*), with an increase in the number of CK13-positive and a decrease in the number of CK14-positive cells in FBS-supplemented medium. Western blot analysis demonstrated a 17-fold increase in CK13 expression and a 1.6-fold decrease in CK14 expression in urothelial cells propagated in medium supplemented with FBS and calcium, relative to control cultures maintained in KSFMc (Fig. 3*B*).

Functional Properties of Cultured Human Urothelial Cells

Electrophysiological properties. The TER of the urothelial cell cultures was significantly affected by the medium in which the NHU cells had been propagated. Cultures established and maintained in KSFMc exhibited a mean TER of 18.5 ± 2.4 Ω·cm² (Table 3). When cultures were switched to 2 mM calcium KSFMc, the mean TER increased, but not significantly, to 49.4 ± 8.9 Ω·cm². Urothelial cells passaged into KSFMc supplemented with FBS and either maintained in this medium or transferred to KSFMc supplemented with FBS and calcium, exhibited a significantly increased TER of 2,509.9 ± 172.2 Ω·cm² ($P < 0.0001$) and 3,023.4 ± 564.4 Ω·cm² ($P < 0.001$), respectively, relative to the KSFMc control; there was no statistical difference between these two TER values.

Transurothelial sodium ion transport. Transcellular transport of sodium ions was demonstrated in NHU cell cultures propagated in KSFMc supplemented with FBS and adjusted to 2 mM calcium. When added to the basal aspect of the culture, amiloride had no effect on the transepithelial potential difference or short-circuit current (Fig. 4*A*). By contrast, apically applied amiloride decreased both measured electrophysiological parameters (Figs. 4 and see 6*A*), suggesting that the urothelial cells transferred sodium ions transcellularly via an apical membrane-restricted amiloride-sensitive ion channel. The change in the short-circuit current induced by amiloride was dose dependent, and the inhibition constant for amiloride was 340 nM (Fig. 5).

The role of Na^+-K^+-ATPase in sodium ion transport across in vitro propagated urothelial cultures was investigated using ouabain. The addition of ouabain to the apical aspect of the cultures had minimal effect on the transepithelial potential difference or the short-circuit current (Fig. 6*C*). However, addition of ouabain to the basal side of the cultures markedly reduced both of the electrophysiological parameters (Fig. 6*D*), suggesting that sodium ions were transported across the basolateral membrane via an active transport mechanism involving Na^+-K^+-ATPase.

Urea and water permeability of human urothelial cell cultures. The mean diffusive permeability of urea through the urothelial cell cultures propagated in KSFMc and KSFMc supplemented with calcium and/or FBS ranged from 2.7×10^{-6} to 14.5×10^{-6} cm/s (Fig. 7). The diffusive permeability of urea through the urothelial cell cultures propagated in KSFMc supplemented with calcium was significantly less than that recorded for the cultures maintained in KSFMc ($P < 0.05$) and KSFMc supplemented with FBS ($P < 0.01$). There was no statistical difference in the urea permeability of cultures propagated in KSFMc supplemented with calcium and those maintained in KSFMc supplemented with calcium and FBS.

Table 2. *Cytokeratin profile in native and cultured urothelial cells*

		Cultured Cells				
Cytokeratin	Normal Urothelium	0.09 mM Ca^{2+} serum-free KSFMc	2 mM Ca^{2+} serum-free KSFMc	0.2 mM Ca^{2+} 5% FBS KSFMc	2 mM Ca^{2+} 5% FBS KSFMc
8	++	++	++	++	++
13	++	+	+	++	++
14	−	++	++	+	+
17	++	++	++	++	++
18	++	++	++	++	++
19	+	++	++	++	++
20	+	−	−	−	−

The immunofluorescence reaction was scored from negative (−) to positive (+++): −, <1% of cells immunopositive; +, 1–50% of cells immunopositive; ++, 50–100% of cells immunopositive.

The diffusive permeability of water through the urothelial cell cultures propagated in KSFMc supplemented with calcium was significantly less than that for the cultures maintained in KSFMc plus FBS ($P < 0.05$).

Dextran permeability of human urothelial cell cultures. The culture medium had a significant effect on the dextran permeability of the urothelial cultures. Relative to the cells propagated in KSFMc, the urothelial cells switched to serum-supplemented medium had a significantly lower permeability to both species of dextran ($P < 0.01$; Fig. 8*A*). In addition, urothelial cells cultured in calcium-supplemented medium exhibited a lower permeability to 4,400, but not 9,500, molecular weight dextran. There was no difference in permeability between cells propagated in serum-supplemented medium and those cultured in KSFMc supplemented with both calcium and serum.

Table 3. *Transepithelial electrical resistance of urothelial cultures*

	Cultured Cells:			
	0.09 mM Ca^{2+} serum-free KSFM	2 mM Ca^{2+} serum-free KSFM	0.2 mM Ca^{2+} 5% FBS KSFMc	2 mM Ca^{2+} 5% FBS KSFMc
TER, $\Omega \cdot cm^2$; Number of cell lines	19.5±2.4 7	49.4±8.9 8	2569±172 18	3023.4±564 7

Values are means ± SE. KSFMc, keratinocyte serum-free medium complete; TER, transepithelial electrical resistance. For each independent cell line at least 2 cell cultures were assessed per medium condition.

DISCUSSION

Since the first serial cultivation of NHU cells in vitro (34), considerable progress has been made to improve propagation techniques, identify gene and antigenic markers of urothelial phenotype, and demonstrate that cultured urothelial cells retain the capacity to differentiate (reviewed in Ref. 38). However, with few exceptions (28, 40), there has been little focus on developing functional urothelial tissue equivalents from propagated cells. The majority of studies have used primary cultures of nonhuman mammalian urothelium to demonstrate aspects of differentiated urothelial tissue function (42), relying on preexisting rather than de novo differentiation.

This study has investigated the functional potential of NHU cells following removal from the disciplined hierarchy of an in situ tissue and propagated as highly proliferative monocultures in vitro. These cells have previously been shown to retain the capacity to express genes and proteins associated with terminal urothelial differentiation (44), but their capacity to form an integrated functional tissue has not been assessed. As it is possible to propagate large numbers of NHU cells from small surgical biopsies (19), the potential to generate a functionally equivalent urothelium from these cells has important implica-

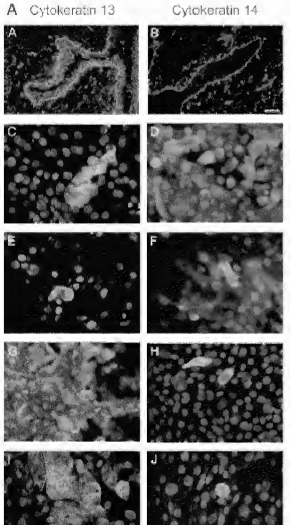


Fig. 3. Cytokeratin (CK)13 and CK14 profile of native and cultured human urothelium. *A*: cryostat sections of human ureter demonstrating labeling for CK13 (*A*; green), CK14 (*B*; green), and the basement membrane constituent laminin (*A* and *B*; red). Laser confocal micrographs of cultured urothelial cells (*C–J*) labeled for CK13 (green) and CK14 (green) and propidium iodide-stained cell nuclei (red) and propagated in KSFMc (*C* and *D*), KSFMc supplemented with calcium (*E* and *F*), FBS (*G* and *H*), and FBS plus calcium (*I* and *J*) are shown. Note increased CK13- and decreased CK14-expression by cells cultured in FBS-supplemented media. Scale bar = 50 μm. *B*: Western blot analysis demonstrated a 17-fold increase in CK13-expression (*left*) and a 4.6-fold decrease in CK14-expression (*right*) in urothelial cells propagated in KSFM supplemented with FBS and calcium (KSFM+) relative to cultures maintained in unsupplemented medium (KSFM).

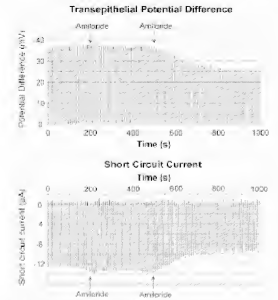


Fig. 4. Urothelial membrane localization of amiloride-sensitive sodium ion channels. The presence and location of sodium ion channels within cultured urothelial cells were investigated using the inhibitor amiloride, while monitoring was done of the transurothelial potential difference (upper) and short-circuit current (bottom). When amiloride (250 nM) was added to the basal aspect of the culture at 200 s, it had no measurable effect on either of the measured electrophysiological parameters. In contrast, when added to the apical compartment at 500 s, amiloride decreased both parameters, suggesting that the amiloride-sensitive sodium channel was restricted to the urothelial apical membrane. Data are representative of 3 independent experiments.

tions for tissue engineering and the development of models for the study of the physiological and pharmacological properties of human urothelium.

According to the definition that leaky epithelia typically have a TER <500 Ω·cm², whereas "tight" epithelia have resistances >500 Ω·cm² (11), mammalian urothelium is regarded as a tight epithelium with low ionic permeability. Although not documented, we anticipated that the TER of human urothelium would be of a similar magnitude to that of other mammalian species, as human bladder tissue has comparable permeability properties (43). This study has demonstrated for the first time that it is possible to develop a urothelium from propagated NHU cells that exhibits a high TER (>3,000 Ω·cm²), thus establishing that cultured NHU cells retain the capacity to reestablish the barrier properties of urothelium in situ.

Perrone and colleagues (33) also assessed the electrophysiological properties of human urothelial cells in culture, but they achieved a mean TER of only 576 Ω·cm², an order of magnitude below that recorded in the present study. Whereas NHU cell lines in this study were obtained from patients with no history or evidence of urothelial pathology, Perrone and

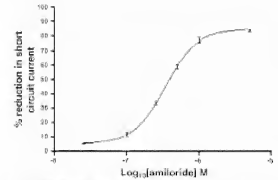
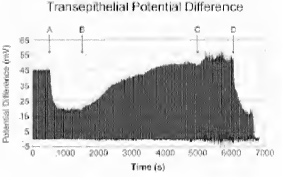


Fig. 5. Dose-response relationship between amiloride and urothelial short-circuit current. Amiloride added to the apical aspect of urothelial cultures propagated in 2 mM Ca^{2+} KSFMdc supplemented with 5% FBS resulted in a dose-dependent decrease in short-circuit current. The inhibition constant for amiloride was 330 nM; $n = 3$.



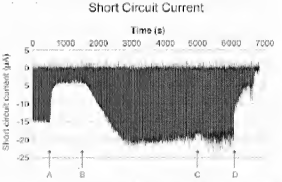


Fig. 6. Transcellular sodium ion transport by urothelial cells propagated in KSFMdc supplemented with FBS and calcium adjusted to 2 mM. The addition of 1 mM amiloride to the apical aspect of the urothelial culture (A) inhibited apical sodium ion entry and significantly decreased the transepithelial potential difference and short-circuit current ($P \leq 0.05$) with a subsequent increase in the TER (from 2,759 ± 226 to 4,464 ± 1,006 Ω·cm², $n = 2$). Apical sodium ion transport was increased by addition of 185 nM nystatin to the apical chamber at 1,500 s (B). The addition of 100 mM ouabain to the apical chamber at 5,000 s (C) had a minor effect on the measured parameters. However, when added to the basal aspect of the urothelial culture at 6,000 s, ouabain (D) inhibited basolateral membrane ion transport, as suggested by the marked decrease in the potential difference and short-circuit current. Data are representative of at least 3 independent experiments.

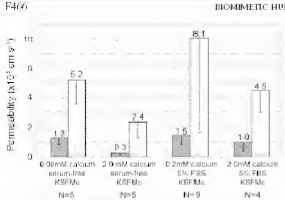


Fig. 7. Diffusive permeabilities of urothelial cell cultures. Diffusive permeabilities of urothelial cell cultures (mean+SD) to radiolabeled urea (grey bars) and water (open bars) are shown.

colleagues generated an immortalized cell line from an individual with interstitial cystitis. Thus the difference in results may be due to the functional capacity of the cells being compromised by SV40T immortalization and/or other differences in culture conditions. Nevertheless, it has been proposed that increased urothelial permeability has a role in the pathophysiology of interstitial cystitis (32). Thus the difference in TER values obtained in this and Perrow's study (33) could be due to an inherent dysfunction in the urothelium in interstitial cystitis and indicates the potential value of in vitro models for studying interstitial cystitis and other such associated conditions.

Mammalian transurothelial sodium ion transport, which has been demonstrated both in vivo (8) and in vitro (24), may have important physiological roles in sodium homeostasis (26) and bladder sensation (19). The hydrostatic pressure gradient across urothelial tissue influences transurothelial sodium ion flux and extracellular release of ATP (10). Through interaction with purinergic P2Xs ion channels, extracellular ATP acts as a neurotransmitter, modulating the afferent limb of the micturition reflex (5). Thus the urothelium is implicated as a sensor and transmitter of information from the physical environment, but the precise nature of these interactions, their role in modulating cellular processes and relevance to dysfunctional uropathies are largely unknown (3, 41), particularly in humans. This study has shown that NHU cells in vitro transport sodium ions transcellularly via apical membrane-restricted sodium ion channels and the Na^+-K^+-ATPase ion pump in the basolateral membrane. In addition, the measured IC_{50} for amiloride inhibition of the short-circuit current was 340 nM, comparable to that previously reported for native rabbit urothelium (10, 27). Thus the cell culture model described in this study is an ideal and practical tool for investigating physiological mechanisms in normal human urothelium.

It was demonstrated that the phenotype of NHU cells in vitro is influenced by the exogenous calcium concentration of the growth medium. However, unlike previous studies that used comparable culture methodologies (19, 37), urothelial cells were shown to undergo stratification in low-calcium conditions, but only when grown on a permeable growth surface. The induction of cellular stratification may have been precipitated by the composition and architecture of the permeable membrane (13), and/or due to membrane facilitation of improved nutrient exchange through the basal cell layer (14).

Analysis of cytokeratin isotype expression suggested that the differentiation states of the urothelial cells was also influenced by the culture conditions. NHU cells in culture express CK8, CK17, CK18, and CK19, all characteristic of native urothelium (19, 37). However, in agreement with previous reports, NHU cells propagated in KSFMc showed downregulation of CK13 in favor of CK14, a CK isotype associated with squamous metaplasia (15), suggesting that in culture, NHU cells switch to a squamous differentiation "program" (45). On transfer into serum-supplemented conditions, the NHU cells readopted a transitional cell phenotype, demonstrating the reversibility of the squamous phenotype of human urothelial cells in vitro.

Although the model described here showed many of the functional and morphological features of normal urothelium, it is still not complete. The study has revealed that NHU cells propagated in vitro can form a partial permeability barrier to water and urea. However, even in the best case, the permeability to water and urea is greater than expected from in vivo measurements (8). In a recently described knockout mouse, deletion of the UPIIIa gene led to incomplete AUM plaque formation and increased water permeability, despite maintenance of the same TER (18). The lack of AUM plaques in this study suggests that despite a transitional cell CK profile, full terminal cytodifferentiation was not attained, thus potentially explaining the measured permeability values. Further modification to the culture system may be required, for example, the introduction of PPARγ agonists (44, 45), to achieve the later stages of urothelial maturation. In static organ culture, a loss of urothelial differentiation with time was suggested to be due to an absence of urine-derived factors or mechanical stimulation (35), and it may be that functional stimulation of cultures will contribute to urothelial maturation.

Terminal junctional complexes, positioned between the umbrella cells, also contribute to urothelial barrier function, by limiting flux via the paracellular route. There is mounting evidence that the molecular composition of the tight junction defines the barrier properties of different epithelial tissues (43). The constitution of mammalian urothelial tight junctions has begun to be elucidated (1), but that of human urothelium remains to be documented. In the present study, the localiza-

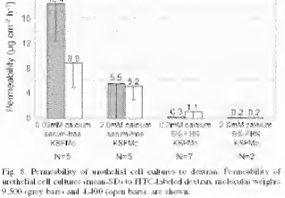


Fig. 8. Permeability of urothelial cell cultures to dextran. Permeability of urothelial cell cultures (mean+SD) to FITC-labeled dextran, molecular weights 9,500 (grey bars) and 4,400 (open bars), are shown.

tion of the tight junction components was similar to that of native tissue, irrespective of the culture medium in which the cells were propagated, suggesting that the documented differences in the permeability properties of the NHU cell cultures was not due to aberrant tight junction formation.

In conclusion, this study has established a methodology to generate a confluent epithelial tissue from in vitro propagated NHU cells that demonstrates many of the functional and phenotypic properties of native urothelium. It clearly demonstrates that NHU cells are not compromised by in vitro propagation, but they retain the capacity to contribute to a functional, ion-transporting epithelium. The full potential of NHU cells to recapitulate the properties of a urinary barrier epithelium will need to be determined by further study, for example, by investigating the expression and function of receptors implicated in sensory mechanisms, such as TRPV1 and TRPM8 (39). However, we feel that the biomimetic human urothelium has a role in dissecting mechanisms involved in normal human urothelial cell physiology and dysfunctional bladder syndromes and has the potential to generate pertinent urothelial facsimiles for bladder tissue engineering (6).

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APPENDIX C

With respect to serum, human and rat urothelial cells respond in opposite fashions, see figure 1 for evidence. In culture Ki67 can be used as a proliferation marker for urothelial cells. Normal rat urothelial cells (NRU) show low proliferation in serum-free medium but higher levels in serum whereas normal human urothelial (NHU) cells show the opposite; reduced proliferation in serum. H33258 is a nuclear stain which shows the total number of cells in each field of view

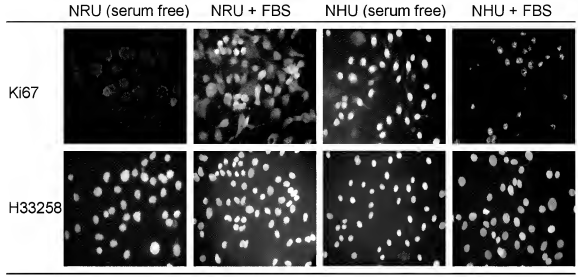

Fig.1 – Immunofluorescent labeling of Ki67, a cell cycle stage marker, in normal rat urothelial (NRU) and normal human urothelial (NHU) cells in the presence and absence of fetal bovine serum (FBS). These micrographs show the expression levels of Ki67 are always the complete opposite in NHU and NRU cells. H33258 is a DNA intercalating dye used to illustrate total cell number in a field of view.

APPENDIX D

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Trans-Species Comparison of PPAR and RXR Expression by Rat and Human Urothelial Tissues

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ABSTRACT

Because some investigational peroxisome proliferator-activated receptors (PPAR) agonists cause tumors in the lower urinary tract of rats, we compared normal human and rat urothelium in terms of PPAR and retinoid X receptor (RXR) expression and proliferation-associated phenotypes. In situ, few human but most rat urothelial cells were Ki67 positive, indicating fundamental differences in cell cycle control. Rat and human urothelia expressed all 3 PPAR and the RXRα and RXRβ isoforms in a predominantly nuclear localization, indicating that they may be biologically active. However, immunolocalization differences were observed between species. First, whereas PPARα and PPARβ/δ were expressed throughout the human bladder or ureteric urothelium, in the rat urothelium PPARα was primarily, and PPARβ/δ exclusively restricted to superficial cells. Second, RXRβ was restricted to intermediate and superficial layers of the human urothelium but tended to be absent from the rat superficial cells. Third, PPARγ expression was present throughout the urothelia of both species but was most intense in the superficial human urothelium. Species differences were also observed in the expression of PPAR and RXR isoforms between cultured rat and human urothelial cells and in the smooth muscle. Our findings highlight the unique coexpression of multiple PPAR and RXR isoforms by urothelium and suggest that species differences in PPAR function between rat and human urothelia may be explored in an in vitro setting.

Keywords: bladder; Ki67; lower urinary tract; PPAR; RXR; urothelium.

INTRODUCTION

Peroxisome proliferator-activated receptors (PPAR) are nuclear hormone receptors, which function as transcription factors to regulate a diverse range of functions (Kersten et al., 2000). Activation of PPAR results in heterodimerization with the 9-cis retinoic acid receptor (RXR), which binds to peroxisome proliferator response elements (PPRE) to activate transcription of target genes. Three PPAR isoforms exist, which are products of distinct genes. These isoforms, termed PPARα, PPARβ/δ, and PPARγ, vary in their tissue distribution and transcriptional activities (Dreyer et al., 1992; Kersten et al., 2000).

Selective PPARα ligands (fibrates) are used clinically as antilipidemic agents, and selective PPARγ agonists (thiazolidinediones; TZDs) are used as insulin sensitizers (Staels and Fruchart, 2005; Leiter, 2006). Selective PPARβ/δ agonists are also being explored for treatment of type II diabetes (Chang et al., 2007). Capitalizing on the beneficial effects of selective PPAR agonists in diabetes, dual-acting PPARα/γ agonists (-glitazars) have been developed, which exhibit improved insulin sensitivity and lipid lowering effects over subtype-specific agonists (Lohray et al., 2001; Brand et al., 2003; Larsen et al., 2003; Ye et al., 2003; Saad et al., 2004; Pickavance et al., 2005).

The continued development of TZDs and dual-acting "-glitazars" has been complicated by carcinogenic effects in rodents (El-Hage, 2004; Cohen, 2005). These included hemangiosarcomas, liposarcomas, hepatomas, and transitional cell carcinomas in the urothelium of the urinary bladder/renal pelvis (El-Hage, 2004). PPAR agonists tested to date have all shown to be nongenotoxic (El-Hage, 2004). Thus, the cancers seen in PPAR agonist-treated rodents likely arise through nongenotoxic mechanisms. Accordingly, the hepatocarcinogenesis induced by PPARα agonists in rodents has been shown to be mediated by the murine PPARα (Peters et al., 2005). Furthermore, the hepatocarcinogenic effect in rodents is not of human relevance, due to structural differences between human and mouse PPARα proteins and functional differences between the human and mouse liver (Morimura et al., 2006).

Dual-acting "-glitazars" appear more potent than PPARγ agonists at inducing tumors associated with the lower urinary tract (LUT) in rats. Some studies suggested an indirect effect, in which bladder cancer resulted from regenerative responses to damage induced by precipitated urinary deposits in male rats (Cohen, 2005; Dominick et al., 2006; Tannehill-Gregg et al., 2007). However, others have suggested a direct effect of PPAR agonists on the rat bladder epithelial (urothelial) lining, based on rapid changes in urothelial gene expression and intracellular signaling, as well as early urothelial hypertrophy in treated rats (Egerod et al., 2005; Oleksiewicz et al., 2005).

To discriminate between direct and indirect PPAR agonist effects, knowledge about PPAR isoform expression in the rat

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Abbreviations: CK, cytokeratin; FDA, Food and Drug Administration; FITC, fluorescein isothiocyanate; KSFM, keratinocyte serum free medium LUT, lower urinary tract; NHU, normal human urothelial; NRU, normal rat urothelial; PPAR, peroxisome proliferator-activated receptors; PPRE, peroxisome proliferator response element; RXR, retinoid X receptor; TZDs, thiazolidinediones; UP, uroplakin.

urothelium is required. Furthermore, to extrapolate from the rat findings, it may be helpful to understand the equivalence between the rat and human urothelium with respect to the expression of receptors and response to PPAR signaling. As the first step toward this aim, we have compared the expression and localization of the known PPAR and RXR receptors in normal rat and normal human urothelial tissues in situ and in vitro. Furthermore, we have extended the previously described method for culturing normal human urothelial (NHU) cells to rats (Southgate et al., 1994; Southgate et al., 2002) and compared PPAR and RXR receptor localization patterns between short-term cultures of rat and human urothelial cells.

Materials and Methods

Antibodies

Primary antibodies used were selected as cross-reacting with human and rat tissues from the suppliers' datasheets. The only exceptions were the antibodies against human PPARα and β/δ, which were raised against highly conserved peptides and in preliminary studies were found to show appropriate immunolabeling patterns on rat and human tissues. Controls were included in all immunochemistry experiments and included use of no primary and irrelevant primary antibody controls.

PPARα rabbit affinity–purified antipeptide antibody from Affinity Bioreagents (Golden, CO; catalogue number PA1-822A) was used at 1 μg.ml^{-1} for immunohistochemistry and 5 μg.ml^{-1} for immunofluorescence.

PPARβ/δ rabbit affinity purified antipeptide antibody from Affinity Bioreagents (catalogue number PA1-823A) was used at 1 μg.ml^{-1} for immunohistochemistry and 5 μg.ml^{-1} for immunofluorescence.

PPARγ mouse monoclonal antibody clone E8 from Santa Cruz Biotechnology (supplied by Autogen Bioclear, Colne, UK; catalogue number SC-7273) was used at 100 ng.ml^{-1} for immunohistochemistry. PPARγ rabbit monoclonal clone 81B8 from Cell Signaling Technology (supplied by New England Biolabs UK, Hitchin, UK; catalogue number 2443) was used at 500 ng.ml^{-1} for immunofluorescence.

RXRα rabbit affinity–purified antipeptide antibody code D-20 from Santa Cruz Biotechnology (catalogue number SC-553) was used at 100 ng.ml^{-1} for immunohistochemistry and 5 μg.ml^{-1} for immunofluorescence.

RXRβ rabbit immunoglobulin from Upstate Biotechnology (Chandlers Ford, UK; catalogue number 06-527) was used at 625 ng.ml^{-1} for immunohistochemistry. RXRβ affinity-purified antipeptide antibody code C-20 from Santa Cruz Biotechnology (catalogue number SC-831) was used at 4 μg.ml^{-1} for immunofluorescence.

Cytokeratin 20 (CK20) mouse monoclonal clone Ks20.8 from Novacastra (Newcastle Upon Tyne, UK; catalogue number NCL-CK20) was used for immunohistochemistry at 5 μg.ml^{-1}

Cytokeratin 7 (CK7) mouse monoclonal clone LP1K, a gift from Cancer Research UK (London), was used at 1:2000 for immunohistochemistry.

UPIIIa mouse monoclonal clone AU1 from Progen Biotechnik (Heidelberg, Germany; catalogue number 651108) was used at 1:100 for immunohistochemistry.

Ki67 mouse monoclonal clone MM1 from Novocastra (catalogue number NCL-L-Ki67) was used at 500 ng.ml^{-1}.

Tissues

Normal Human Urothelium

The collection of surgical specimens was approved by the relevant Local Research Ethics Committees and had full, informed patient consent. Surgical specimens of normal urothelium were obtained from patients with no histological evidence of urothelial dysplasia or malignancy (Table 1). Tissues were collected in transport medium, consisting of Hank's balanced salt solution (HBSS) containing 10 mM HEPES pH 7.6 and 20 KIU aprotinin (Trasylol, Bayer plc, Newbury, UK), as described previously (Southgate et al., 1994; Southgate et al., 2002). Tissues were either fixed in 10% formalin for 24 hours and transferred to 70% (v/v) ethanol before processing into paraffin wax for immunohistology or used to establish finite cell lines of normal human urothelial (NHU) cells.

NHU Cell Culture

NHU cell lines of finite lifespan were established from resection specimens of ureteric urothelium and maintained in complete keratinocyte-serum free medium (KSFM(c) consisting of KSFM supplemented with bovine pituitary extract, epidermal growth factor at the manufacturer's recommended concentrations (Invitrogen, Paisley, UK), and cholera toxin (30 ng.ml^{-1}, Sigma Aldrich, Poole, UK). The preparation, maintenance, and characterization of NHU cell cultures has been previously detailed, including comparison of cultures derived from ureteric and bladder sources (Southgate et al., 1994; Southgate et al., 2002). NHU cell lines were used between passages 2 and 5.

Normal Rat Urothelium

Normal rats were anesthetized with medical grade CO_2 and euthanized by cervical dislocation, in accordance with UK Home Office regulations. Thereafter, urinary bladders were rapidly excised and collected either into 10% (v/v) formalin for 24 hours and transferred to 70% (v/v) ethanol before processing into paraffin wax or into ice-cold transport medium for cell culture. The rodent tissues used in this study are detailed in Table 1.

Rat Urothelial Cell Culture

Under aseptic conditions, rat bladders from male 9-month Wistar rats were carefully dissected into smaller pieces and placed in stripping medium consisting of HBSS (without Ca^{2+} or Mg^{2+}) with 10 mM HEPES pH 7.6, 20 KIU Trasylol, and 0.1% (w/v) EDTA. After 4 hours rotation at 37°C, the urothelium was carefully stripped as sheets from the underlying stroma.

TABLE 1.—Details of human and rat tissue specimens.

Sample ID	Tissue	Age	Sex	Origin
Y454		NR	NR	Nephrectomy
Y686		86	M	Nephrectomy
Y712	Normal	64	F	Nephrectomy
Y718	human ureter	NR	NR	Nephrectomy
Y744		56	M	Nephrectomy
Y779		NR	NR	Nephrectomy
Y835		77	M	Nephrectomy
Y732	Normal	38	M	Renal transplant
Y804	human bladder	NR	NR	Prostatectomy
Y850		68	M	Cystectomy
Y864		59	M	Prostatectomy
RB11		8 weeks	M	
RB16	Rat bladder (W)	8 weeks	M	n/a
RB17		8 weeks	M	
RB18		5 weeks	M	
RB19	Rat bladder (SD)	5 weeks	M	n/a
RB20		5 weeks	M	

W = Wistar strain (a hooded), SD = Sprague Dawley strain (Charles River Laboratories Ltd, Margate, UK); M = male; F = female; NR = not reported.

collected by centrifugation at 400 g for 5 minutes, resuspended in HBSS containing 250 mg.ml^{-1} collagenase type IV (from *Clostridium histolyticum*; Sigma Aldrich) and incubated at 37°C for 30 minutes. Urothelial sheets were collected and disaggregated with gentle pipetting, and following centrifugation, the urothelial cells were resuspended in KSFMc and seeded at 0.25 × 10^6 cells per 25 cm^2 Primaria® tissue culture flasks (BD Biosciences, Oxford, UK). Growth medium was changed every 3 days, and cultures were passaged at 80% to 90% confluence, as described for NHU cells (Southgate et al., 1994; Southgate et al., 2002). As described previously for NHU cell cultures, the separation of the urothelium from the basement membrane as an intact cell sheet limits the potential for contamination of the primary culture by stromal-derived cells, and the use of a serum-free medium developed for keratinocyte cell culture further promotes epithelial but not stromal cell growth (Southgate et al., 1994; Southgate et al., 2002). Thus, the cultures are of urothelial derivation (Nicholls et al., in preparation). Each primary culture of normal rat urothelial (NRU) cells was established from 6 pooled bladders, which were seeded initially into 2 × 25 cm^2 flasks and used between passages 1 and 3.

Immunofluorescence Microscopy

Cultured human or rat urothelial cells were grown to 70% to 80% confluence on 12-well glass slides, fixed in a 1:1 mixture of methanol and acetone, air-dried, and incubated overnight at 4°C with titrated primary antibodies or no antibody controls. After extensive washing, slides were incubated in Alexa 488-conjugated goat anti-mouse IgG (5 μg.ml^{-1}; Invitrogen) or goat anti-rabbit IgG (4 μg.ml^{-1}; Invitrogen) for 30 minutes at ambient temperature, before washing in PBS containing 0.25% Tween 20. 0.1 μg.ml^{-1} Hoechst 33258 (Sigma Aldrich) was added to the last wash to visualize nuclei. Slides were examined under epifluorescence illumination on an Olympus BX60 microscope.

Immunohistochemistry

Sections (5 μm) of paraffin wax–embedded tissue were dewaxed in xylene and rehydrated through ethanol. Endogenous peroxidase activity was blocked by incubation with 3% (v/v) hydrogen peroxide for 10 minutes. Antigen retrieval was performed by digestion of sections for 1 minute in 0.1% (w/v) trypsin in 0.1% (w/v) $CaCl_2$, pH 7.6, followed by boiling for 10 minutes in 10 mM citric acid buffer, pH 6.0 in a microwave oven. Trypsinization was not required for anti-PPARα, RXRα, RXRβ, UPIIIa, and Ki67 antibodies. Endogenous avidin-binding sites were blocked using an avidin/biotin blocking kit (Vector laboratories, Peterborough, UK) according to the manufacturer's protocol. A 5-minute blocking step was included to prevent nonspecific binding of secondary antibodies (using either 10% rabbit or goat serum in 10 mM Tris-buffered saline, pH 7.6). Slides were incubated with primary antibody overnight at 4°C, washed 3 times in TBS, and incubated with appropriate secondary antibody for 30 minutes at room temperature (biotinylated rabbit anti-mouse at 1:400 and goat anti-rabbit at 1:800, Dako Cytomation Ltd, Ely, UK).

To improve the sensitivity of detection of PPAR and RXR antigens, tyramide-based catalyzed signal amplification was used (Dako Cytomation Ltd; Stahlschmidt et al., 2005). All other primary antibodies were visualized by avidin-biotin-peroxidase detection using a StreptABComplex kit (Dako Cytomation Ltd) according to the manufacturer's instructions, with 3,3'-diaminobenzidine as chromogen (Sigma Aldrich). Slides were counterstained with Mayer's hematoxylin, dehydrated through ethanol into xylene, and mounted in DPX (Fisher, Loughborough, UK).

RESULTS

Urothelial Morphology

Histological integrity and differentiated phenotype of human and rat urothelia was confirmed by cytokeratin and uroplakin immunohistochemistry, as illustrated in Figure 1. CK7 was expressed by all layers of both human and rat urothelia. UPIIIa was present along the superficial luminal edge of all human and rat urothelial samples tested and further extended into the intermediate cell layers in the rat urothelium only. The observation that uroplakin expression is localized at the apical edge of the superficial cell in the human urothelium, but is less restricted in the rodent urothelia, is in agreement with previous reports (Mo et al., 2005). The intermediate filament protein, CK20, was expressed by all human and rat tissue samples, with expression limited to the superficial cells.

To determine the proliferative status of the urothelia, the expression of Ki67, a nuclear proliferation marker present during active cell cycle (G1, S, G2, and M phase) and absent in resting (G0) cells was assessed. In the human urothelium, very few Ki67-positive cells were observed in either the ureter ($n = 7$) or bladder ($n = 4$), and, where present, labeling was restricted to a few, predominantly basal cells (Figure 1). By contrast, the rat urothelium exhibited strong nuclear Ki67 labeling of all basal and intermediate cells, whereas superficial cells were negative.

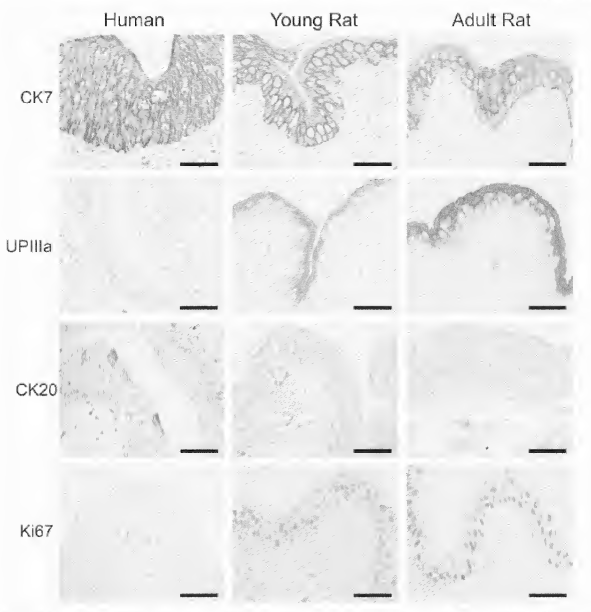

FIGURE 1.—Trans-species expression of markers of urothelial tissue integrity by immunohistochemistry. Expression of cytokeratin 7 (CK7), uroplakin IIIa (UPIIIa), and cytokeratin 20 (CK20) was examined by immunohistochemistry in paraffin wax–embedded urothelial tissue samples obtained from human and young (5 weeks) and mature (9 months) Sprague Dawley rats. The expression of the proliferation marker Ki67 was also examined. Scale bar = 50 μm.

The same Ki67 expression pattern was seen consistently in both Sprague Dawley and Wistar rat strains and in both young (5–8 weeks; $n = 4$) and mature (9 months; $n = 2$) rats (see Table 1).

Expression and Distribution of PPAR and RXR in Rat and Human Urothelial Tissues

The expression and localization patterns are summarized in Table 2 and illustrated on tissue sections by immunohistochemistry for urothelium (Figure 2) and smooth muscle (Figure 3) and in urothelial cell cultures by immunofluorescence (Figure 4). No differences were noted in PPAR or RXR immunolocalization on the human urothelium from the bladder ($n = 4$) or ureter ($n = 3$).

PPARα

The human urothelium displayed prominent nuclear PPARα labeling throughout, with weaker diffuse labeling of the cytoplasm

TABLE 2—Summary of immunohistochemical distribution of PPAR and RXR isoforms in human and rat urothelial tissues and cells.

	PPARα	PPARβ/δ	PPARγ	RXRα	RXRβ
Human					
Urothelium in situ bladder and ureter, $n = 11$					
Superficial	n & c	n	n	n	n
Intermediate	n & c	n	n	n	n
Basal	n & c	(n)	n	n	—
NHU cell culture ($n = 3$)	(n & c)	n	nS	n & (c)	(n & c)
Bladder smooth muscle in situ ($n = 4$)	n	c	(n)	n	n*
Ureteric smooth muscle in situ ($n = 7$)	n	c	n	—	n
Rat (Wistar and Sprague Dawley)					
Urothelium in situ ($n = 6$)					
Superficial	n & c	n	n	n	n*
Intermediate	c	—	n	n	n
Basal	c	—	n	n	n
NRU cell culture ($n = 3$)	(n & c)	n	n	(n & c)	n & (c)
Smooth muscle ($n = 6$)	c	—	—	n*	n

n = nuclear, c = cytoplasmic. () = weak expression; bold indicates dominant pattern. * = some cells only. S = confluent cultures. – = negative. PPAR = peroxisome proliferator-activated receptor. RXR = retinoid X receptor. NHU = normal human urothelial. NRU = normal rat urothelial.

The rat urothelium exhibited nuclear PPARα labeling, which was most intense in the superficial cells and accompanied by weak, diffuse cytoplasmic labeling throughout the urothelium. PPARα was also detected in the human bladder and ureteric smooth muscle, where there was intense nuclear and minimal cytoplasmic labeling. Diffuse cytoplasmic labeling was also present in the rat bladder detrusor smooth muscle. Cultured urothelial cells from human and rat origins exhibited weak nuclear and cytoplasmic PPARα immunoreactivity.

PPARβ/δ

In the human urothelium, PPARβ/δ expression was restricted primarily to the nuclei of superficial and intermediate cells, with less intense labeling of the basal cells. In the rat urothelium, PPARβ/δ was restricted almost exclusively to nuclei of superficial cells, with very little if any expression evident in the other urothelial layers. In the human bladder and ureteric smooth muscle, PPARβ/δ immunolabeling was weak, diffuse, and cytoplasmic, with no nuclear component. PPARβ/δ immunoreactivity was absent from the rat detrusor smooth muscle. In cultured cells from both human and rat urothelia, PPARβ/δ immunoreactivity was predominantly nuclear. Within the nuclei, the labeling was intense, punctate, and excluded from nucleolar regions.

PPARγ

Intense nuclear PPARγ immunoreactivity was present in all layers of human and rat urothelia, with no cytoplasmic component. There was a tendency for labeling to be most intense in the superficial cells of the human urothelium. Nuclear PPARγ was also present in the human bladder and ureteric smooth muscle but was completely absent from the rat detrusor smooth muscle. In the human urothelial cell culture, localization of PPARγ varied according to the state of confluency, being primarily nuclear in confluent culture but diffusely cytoplasmic and less nuclear in subconfluent cultures; this was consistent with previous observations (Varley, Stahlschmidt, Lee, et al., 2004). In confluent NHU and in NRU cell cultures, PPARγ showed an intense nuclear localization, which was punctate and excluded from nucleolar regions.

RXRα

Intense nuclear RXRα immunoreactivity was observed in all urothelial cells within human and rat urothelial tissues. Although RXRα expression was negative in smooth muscle from the human ureter, it was nuclear in a majority of cells in the human bladder detrusor smooth muscle. In the rat detrusor smooth muscle, nuclear RXRα expression was detected in a proportion of smooth muscle cells. By immunofluorescence on urothelial cell cultures, RXRα was intense and nuclear in NHU cells but showed weaker nuclear labeling of NRU cells; in both species, there was also diffuse cytoplasmic immunoreactivity.

RXRβ

In the human urothelium, RXRβ was nuclear and present predominantly in the superficial and intermediate cells, with weaker or absent nuclear labeling of the basal cells. Intense nuclear RXRβ was present in rat urothelial cells from basal and intermediate cell layers but absent from many superficial cells. Nuclear RXRβ immunoreactivity was evident in human ureteric and rat bladder smooth muscle but was only expressed by some cells in the human bladder smooth muscle. RXRβ was intensely nuclear and punctate in NRU cell cultures but was less intensely nuclear in NHU cells, where there was also a cytoplasmic component.

Discussion

The transitional epithelium that lines much of the LUT is a mitotically quiescent tissue with a constitutively low rate of turnover, yet it maintains a high regenerative potential in

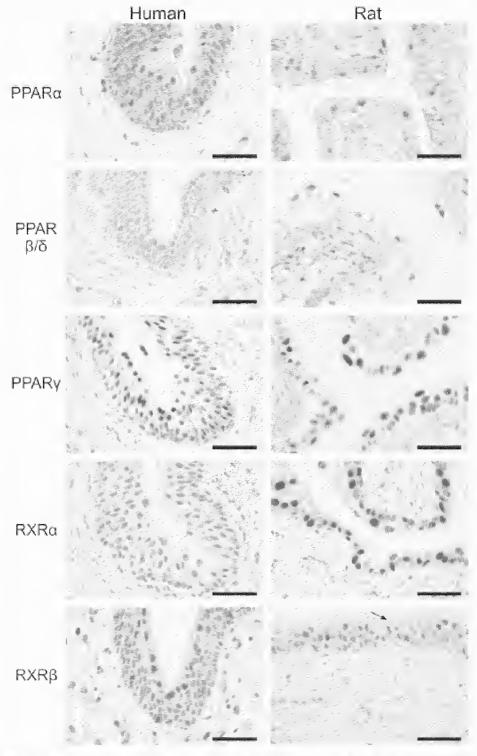

FIGURE 2.—Expression of peroxisome proliferator-activated receptors (PPARs) and retinoid X receptors (RXRs) in human ureteric and rat bladder urothelium by immunohistochemistry. Expression of PPAR and RXR antigens was examined by immunohistochemistry in paraffin wax–embedded urothelial tissues. Arrow indicates occasional RXRβ-negative superficial urothelial cell in rat urothelium. Scale bar = 50 μm.

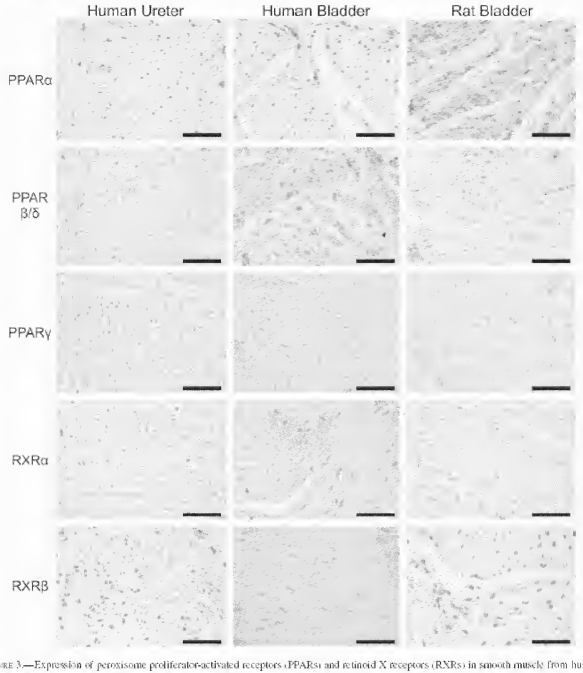

FIGURE 3.—Expression of peroxisome proliferator-activated receptors (PPARs) and retinoid X receptors (RXRs) in smooth muscle from human ureteric and bladder and in rat bladder smooth muscle by immunohistochemistry. Expression of PPAR and RXR antigens was examined by immunohistochemistry in paraffin wax–embedded urothelial tissues. Scale bar = 50 um.

response to a range of insults, such as damage, injury, and infection (Hicks, 1975). Ki67 is a proliferation marker that is synthesized early in G1 phase and is absent from cells that have withdrawn from the cell cycle. Accordingly, in both rat and human urothelia, the terminally differentiated superficial cells that coexpressed UPIIIa and CK20 were Ki67 negative. However, whereas very few human urothelial cells were Ki67 positive, most basal and intermediate rat urothelial cells were Ki67 positive, irrespective of age or strain (Wistar or Sprague Dawley; data not shown). Others have reported lower levels of Ki67 labeling of

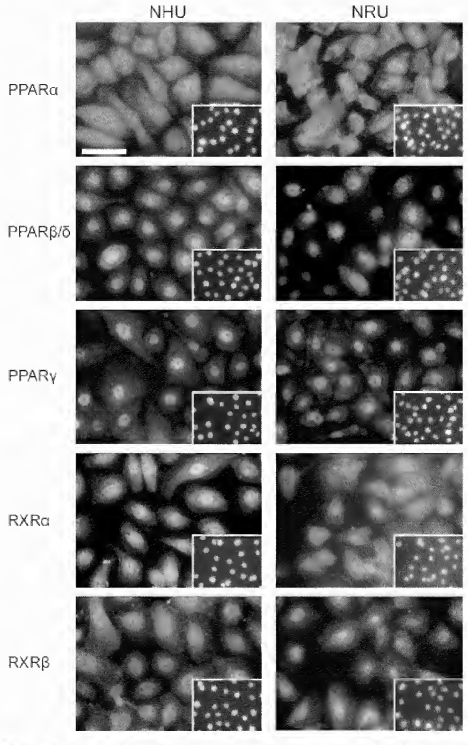

FIGURE 4.—Expression of peroxisome proliferator-activated receptors (PPARs) and retinoid X receptors (RXRs) in cultured human and rat urothelial cells. Indirect immunofluorescence labeling of PPAR and RXR antigens on methanol/acetone-fixed human and rat urothelial cell cultures; scale bar = 50 μm. The smaller insert micrographs show the identical field stained with Hoechst 33258, to indicate positions of nuclei. NHU = normal human urothelial; NRU = normal rat urothelial.

the rat bladder urothelium (Nguyen, 2007). However, our findings are supported by flow cytometric cell cycle analysis of freshly dissociated rat urothelial cells, which revealed an unusual combination of very low S-phase percentages (<1%) and disproportionately high G2/M percentages (9.12%; Kaneko et al., 1984; Oleksiewicz et al., 2005). By contrast, freshly dissociated human urothelial cells exhibited a high G0/G1 population that was released rapidly into cell cycle after seeding in culture (Varley et al., 2005). We believe that it is highly unlikely that the Ki67-positive cells in the rat urothelium are actively proliferating, as rat urothelial cells have a very low S-phase percentage and very low BrdU labeling indices (Oleksiewicz et al., 2005; Dominick et al., 2006). Rather, we suggest that the Ki67-positive cells represent a pool of proliferation-capable cells, providing the well-known rapid regenerative potential of normal urothelium. The difference in Ki67 labeling suggests a fundamental difference in how urothelial regeneration is regulated in the rat and human. It is tempting to speculate that this difference may contribute to the susceptibility of the rat to urolithiasis-mediated bladder cancer (Cohen, 2005).

Chronic activation of PPARγ and PPARα has been implicated in transitional cell carcinoma development in rats, as evidenced by rapid changes in urothelial gene expression and intracellular signaling, as well as early urothelial hypertrophy in treated rats (Egerod et al., 2005; Oleksiewicz et al., 2005). The major prerequisite for this mechanism is urothelial coexpression of PPARγ and PPARα, a nontrivial assumption, as the PPARγ and PPARα isoforms generally exhibit nonoverlapping expression patterns (Chang et al., 2007). In situ hybridization studies have demonstrated transcripts for all 3 PPAR genes in human and rabbit urothelia (Guan et al., 1997), and PPARγ transcripts are also expressed in the mouse urothelium, as well as in the presumptive urothelium of the urogenital sinus (Jain et al., 1998). However, transcript expression may not necessarily relate to expression of functional receptors. In this study, we have shown for the first time that all 3 PPAR isotypes and the RXRα and RXRβ heterodimerization partners are expressed at the protein level in urothelial cells. Thus, in the rat LUT, there appears to be a correlation between PPAR isoform expression patterns and susceptibility to the carcinogenic effect of some PPAR agonists (El-Hage, 2004; Egerod et al., 2005; Oleksiewicz et al., 2005). However, it remains to be determined whether there is a causal effect of PPARs in bladder cancer development in rats.

We have previously demonstrated expression of PPARγ and its heterodimerization partner RXRα in NHU cells (Stahlschmidt et al., 2005; Varley, Stahlschmidt, Smith, et al., 2004) and have reported that PPARγ signaling initiates differentiation of NHU cells (Varley, Stahlschmidt, Lee, et al., 2004; Varley, Stahlschmidt, Smith, et al., 2004; Varley et al., 2006). In the present study, we confirmed expression of PPARγ and RXRα by human urothelium in situ and in vitro. Biological activity of the PPARγ and RXRα transcription factors in the human urothelium in situ was indirectly supported by the exclusively nuclear localization patterns. Furthermore, the finding that the human as well as rat urothelium expresses all 3 PPAR isoforms and both the RXR α and β (but not RXRγ; unpublished data) isoforms supports the hypothesis that PPAR signaling is a key, phylogenetically conserved constituent of urothelial biology. While PPARα and RXRα expression was seen in all urothelial layers in the rat as well as human, expression of PPARγ, PPARβ/δ, and RXRβ appeared to show some correlation with differentiation stage (Table 2), as would be predicted from previous studies in NHU cell cultures (Stahlschmidt et al., 2005; Varley, Stahlschmidt, Lee, et al., 2004; Varley, Stahlschmidt, Smith, et al., 2004; Varley et al., 2006). Intriguingly, rat and human urothelia exhibited some differences in the distribution of PPAR and RXR isoforms (Table 2), the relevance of which is as yet unknown, but which may indicate species-specific differences in urothelial responses to PPAR signaling. The expression and localization of receptors was generally equivalent between urothelia in situ and in vitro, although some differences were noted in the distribution between nuclear and cytoplasmic compartments. This is likely to reflect modulating influences of the different environments, for example, the availability of ligand or the influence of other signaling pathways. For example, we have shown previously that autocrine activation of the epidermal growth factor receptor in subconfluent NHU cell cultures results in phosphorylation of PPARγ and sequestration in the cytoplasmic compartment (Varley, Stahlschmidt, Lee, et al., 2004).

Although RXRs are important regulators of PPAR function, prior to this study, very little was known about the expression of RXRs in the lower urinary tract. We have shown that a major point of difference between human and rat urothelia in situ was in the pattern of expression of RXRα and RXRβ, which were also differentially expressed in vitro. Agonist-bound PPARs heterodimerize with RXRs to bind specific PPREs, activating transcription of target genes (Desvergne and Wahli, 1999; Berger and Moller, 2002). PPARs can form heterodimers with all RXRs, and specific combinations can influence the recognition of target gene promoters (Juge-Aubry et al., 1997; Feige et al., 2006). In our opinion, the observed colocalization between PPARs and RXRs in urothelial cells of humans as well as rats supports a biological function for PPAR signaling in urothelial biology. The significance of RXR isoform expression to differential species responses is at present unknown, but our study raises the possibility that it could be addressed in the in vitro setting.

Finally, it should be mentioned that we observed PPAR and RXR expression in smooth muscle cells of the lower urinary tract, the implication of which is as yet unknown. Two particular points of interest were the differential expression of RXRα and RXRβ by human bladder and ureteric smooth muscle, respectively, and the observation that in both rats and humans, PPARs were more highly coexpressed in urothelium than in smooth muscle, again supporting a unique role for PPAR signaling in urothelial biology (Table 2).

In summary, the present study has described the expression of PPAR and RXR receptors in human and rat urothelium and detrusor smooth muscle. This study has confirmed that the urothelium is a potential target tissue for PPAR signaling and has indicated a number of significant differences in expression and distribution of PPARs and RXRs between species. These differences may underlie a differential response to PPAR agonists via

the assembly and activity of specific PPAR/RXR heterodimers. The differences in expression of the proliferation marker, Ki67, between the species further suggests that rat and human urothelia may respond differentially following an insult/infection. Although PPARγ signaling is implicated in proliferation and differentiation (Varley, Stahlschmidt, Lee, et al., 2004; Varley, Stahlschmidt, Smith, et al., 2004; Varley et al., 2005, 2006), the role of PPARα and PPARβ/δ signaling in the urothelium has not been investigated. In other epithelial tissues, PPARα has been shown to affect hepatocellular proliferation (Shah et al., 2007) and to inhibit vascular smooth muscle cell proliferation (Zahradka et al., 2003, 2006), whereas activation of PPARβ/δ can induce terminal differentiation with concomitant inhibition of cell proliferation in keratinocytes (Kim et al., 2005) and Paneth cells via hedgehog signaling (Varnat et al., 2006). As our study shows that human and rat urothelial cell cultures retain the in situ expression patterns of PPAR and RXR isotypes, an in vitro experimental approach may clarify the role of PPARα and PPARδ signaling in the urothelium and provide a route to bridging the cross-species barrier.

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